

Internal arsenite bioassay calibration using multiple bioreporter cell lines

Published in:

Microbial Biotechnology (2008) 1(2), 149–157, doi:10.1111/j.1751-7915.2007.00011.x

Final submitted and revised version.

Anke Wackwitz^{1*}, Hauke Harms¹, Antonis Chatzinotas¹, Uta Breuer¹, Christelle Vogne², Jan
Roelof van der Meer²

Running title: Bioassay calibration with multiple cell lines

¹UFZ, Helmholtz Centre for Environmental Research, Department of Environmental
Microbiology, 04318 Leipzig, Germany

²Department of Fundamental Microbiology, University of Lausanne, 1015 Lausanne,
Switzerland

* Corresponding author: UFZ, Helmholtz Centre for Environmental Research,
Department of Environmental Microbiology, Permoserstrasse 15, D-04318 Leipzig, Germany.
Tel.: +49 341 235 4460. Fax: +49 341 235 2247. E-mail: anke.wackwitz@ufz.de.

Abstract:

Bioassays with bioreporter bacteria are usually calibrated with analyte solutions of known concentrations that are analyzed along with the samples of interest. This is done since bioreporter output (the intensity of light, fluorescence or colour) does not only depend on the target concentration, but also on the incubation time and physiological activity of the cells in the assay. Comparing the bioreporter output with standardized colour tables in the field seems rather difficult and error-prone. A new approach to control assay variations and improve application ease could be an internal calibration based on the use of multiple bioreporter cell lines with drastically different reporter protein outputs at a given analyte concentration. To test this concept, different *Escherichia coli*-based bioreporter strains expressing either cytochrome c peroxidase (CCP, or CCP mutants) or β -galactosidase upon induction with arsenite were constructed. The reporter strains differed either in the catalytic activity of the reporter protein (for cytochrome c peroxidase) or in the rates of reporter protein synthesis (for beta-galactosidase), which, indeed resulted in output signals with different intensities at the same arsenite concentration. Hence, it was possible to use combinations of these cell lines to define arsenite concentration ranges at which none, one or more cell lines gave qualitative (yes/no) visible signals that were relatively independent from incubation time or bioreporter activity. The discriminated concentration ranges would fit very well with the current permissive (e.g. WHO) levels of arsenite in drinking water (10 $\mu\text{g/L}$).

Introduction

In the recent years, new tools for environmental monitoring have been developed on the basis of so-called bioreporter organisms. Bioreporters are often microbial strains which are genetically modified to produce easily detectable output in response to the presence of specific analytes. Several reviews have been dedicated to this rapidly developing field, exposing details of genetic constructions, type of reporters or analyte detection (Daunert et al. 2000; Leveau & Lindow 2002; Belkin 2003; van der Meer et al. 2004; Harms et al. 2006). The detection system of bioreporter microorganisms is in most cases composed of a simple genetic circuitry, comprising a regulatory system (i.e. regulatory gene plus its cognate promoter) and one or more reporter genes. Commonly used reporter proteins are the chromogenic β -galactosidase (*lacZ*) or β -glucuronidase (*gusA*), luminescent luciferases (*luc* or *luxAB*) or autofluorescent proteins (GFP and variants) (Ivask et al. 2002; Paitan et al. 2004; Werlen et al. 2004; DeAngelis et al. 2005). For quantitative measurements, bioreporter assays need to be calibrated with analyte solutions of known concentrations, which are typically analyzed along with the samples of interest (Hakkila et al. 2004; Harms et al. 2005). The reason for this is that the bioreporter output (colour, luminescence or fluorescence) does not only depend on analyte concentration, but also on assay incubation time and the numbers and physiological activities of the cells in the assay (Jansson 2003; Stocker et al. 2003; van der Meer et al. 2004; Wells et al. 2005). Simple

1 applications like bioreporter field test kits or paper test strips are hampered by the difficulty to
2 compare the resulting colour signals with standardized colour tables. Therefore, a robust and
3 reliable system for internal calibration would be highly desirable.

4 The problem of variable incubation time and uncertain cellular activity could be overcome by
5 the simultaneous use of multiple bioreporter cell constructs, each giving qualitative
6 information about the presence or absence of analyte in a certain concentration range.
7 Bioreporters could be designed and combined in such a way that not the actual individual
8 output but the combination of reacting cell lines indicates the analyte concentration range in
9 unknown samples without the need to measure exact rates or endpoints of colour formation by
10 individual bioreporters (Fig. 1). Although this method would not report exact concentrations,
11 the identification of concentration ranges should be sufficient for many applications, where,
12 for instance, only a distinction of concentrations below and above a predefined permissible
13 level is desired. If in combination with a chromogenic reporter protein this would allow signal
14 interpretation by eye without instrumentation. In the case of arsenic in potable water, a
15 distinction between concentrations below and above the WHO drinking water standard of 10
16 $\mu\text{g/L}$ or the local standard of 50 $\mu\text{g/L}$ in the most arsenic-threatened countries of South-East-
17 Asia would be most desirable.

18 To test this concept, we used two reporter genes to redesign bioreporter bacteria that had
19 previously been developed for the detection of arsenite in drinking water, exploiting the ArsR
20 sensor transcriptional repressor of the natural arsenic resistance of *Escherichia coli* (Stocker
21 et al. 2003). In the presence of arsenite, ArsR loses affinity for its binding site on the DNA the
22 result of which is depression of the *arsR* promoter. Equally effective in derepressing the *arsR*
23 promoter via ArsR is antimonite. In cells which carry the arsenate reductase ArsC, arsenate
24 (at 25% efficiency) and trimethylarsine oxide (at 10% efficiency) will also lead to
25 derepression (Baumann & van der Meer, 2007). In one series of cell lines, the *arsR* promoter
26 was combined with *lacZ* for β -galactosidase. Beta-galactosidase has many advantages and
27 various chromo- and fluorogenic substrates exist to assay LacZ activity (Daunert et al. 2000;
28 Lei et al. 2006). In a second series of cell lines we applied the *ccp* gene. Its product, the
29 cytochrome c peroxidase (CCP) has not frequently been utilised in whole cell bioreporter
30 bacteria so far, but a large variety of *ccp* mutants has been developed which display different
31 catalytic activity for one of its substrates guaiacol (Iffland et al. 2000). The signal outputs of
32 both reporters were thus varied in different ways: for LacZ bioreporters we modified the
33 intervening sequence between *arsR* and *lacZ* in order to modulate *lacZ* expression. For *ccp*
34 we maintained the same reporter synthesis rate, but hypothesized that because of different

catalytic activity of the reporters we would get variable output of the cells at the same arsenite concentration. Our research questions therefore were (i) if both types of reporter constructions would result in different levels of reaction sensitivity to arsenite and (ii) if combinations of such reporter cell lines with variable signal outputs would be suitable to infer arsenite concentration ranges without external calibration and at the required drinking water standards.

Results

β -Galactosidase-based bioreporter assay. Bioassays were performed in 96-well plates to allow spectrophotometrical measurements (Fig. 2) as well as visual signal detection by digital imaging (Fig. 3). For each bioreporter strain the bioassay was performed in triplicate with 15 different arsenite concentrations ranging from 0.2 to 100 $\mu\text{g/L}$. Strain 2245 was very sensitive and produced visible colour from the lowest arsenite concentration (0.2 $\mu\text{g/L}$) with a linear signal increase up to 6 $\mu\text{g/L}$ and a plateau above 10 $\mu\text{g/L}$ (Figs. 2 & 3). Colour development by strain 1595 began at approximately 10 $\mu\text{g/L}$ arsenite and linearly increased at higher concentrations. Both strains had a relatively low background activity in the absence of arsenite. In contrast, strain 1580 was characterized by a high background and little further signal increase toward higher concentrations. Strain 2066 was the least sensitive and gave visible signals only at very high concentrations (around 50 $\mu\text{g/L}$). Visual signal differentiation for neighbouring concentrations was difficult with all strains. The combination of qualitative (yes/no) signals of all four strains appeared suitable for the definition of three arsenite concentration ranges: $<6 \mu\text{g/L}$, $10\text{--}50 \mu\text{g/L}$ and $>50 \mu\text{g/L}$ (Tab. 1A). The quality of the responses (visible colour or not) was independent from incubation time in a window between 4 and 8 hours after induction. They were also independent from cell density for final cell culture turbidities in the assays of between OD_{600} of 0.1 and 1.0 (data not shown).

Cytochrome c peroxidase-based bioreporter assay. In this case, the assays were carried out with colonies exposed to arsenite on agar plates and transferred to paper in order to obtain optimal visual signal interpretation. For each strain the bioassay was performed in four replicates with 10 different arsenite concentrations ranging from 1 $\mu\text{g/L}$ to 5 mg/L . The bioreporter strains 1971, 1981 and 1982 displayed relatively similar responses and produced clearly visible, concentration-dependent signals between 4 and 30 $\mu\text{g/L}$ followed by a plateau (Fig. 4). For the strains 1971 and 1982 a further signal increase could be detected after incubation with 1000 $\mu\text{g/L}$. The background activity in the absence of arsenite was high

1 enough to result in visible spots. Strain 2031 showed no background activity without arsenite,
2 while colour production was clearly visible at 4 µg/L and increased strongly until 20 µg/L.
3 Strains 2332 and 2340 did not respond to low arsenite concentrations (1-10 µg/L) and
4 produced slightly increasing signals between 20 µg/L and 5 mg/L arsenite. The combination
5 of the qualitative signals of these strains appeared to enable the distinction of arsenite free
6 controls from the concentrations ranges 1-10 µg/L and >20 µg/L (Tab. 1B). Similar results
7 were obtained with either twice as many cells per spot or shorter (2 h) and longer incubation
8 (16 h) (Figure S1).

9 **mRNA content.** Reporter mRNA contents of cells exposed to three arsenite concentrations
10 were quantified in order to determine whether differences in signal intensities for the reporters
11 at identical arsenite concentrations were the result of differential transcription or of enzyme
12 synthesis and enzyme activity. Levels of the *ccp* mRNA in the different CCP bioreporter
13 strains were similar after incubation with the same arsenite concentration, but increased as a
14 function of arsenite concentration (Fig. 5A). An exception was strain 1982, which for
15 unknown reasons contained significantly ($p<0.05$) less *ccp* mRNA at the highest arsenite
16 concentration. The level of *lacZ* mRNA in the different reporter strains generally increased as
17 a function of arsenite concentration, but varied between all strains. At 100 µg As/L the levels
18 of *lacZ* mRNA differed significantly ($p<0.05$) between all strains, while at 10 µg As/L the
19 difference was only significant between strain 1580 and the other strains ($p<0.05$). This
20 indicates that the change of the intergenic region between *arsR* and *lacZ* in the different
21 constructs, and the use of two different plasmid vectors for cloning (pMV for 1580 and 1595;
22 pPROBE for 2245 and 2066) did affect the amount of *lacZ* mRNA produced. Notably, *lacZ*
23 expression in strain 1580 without and at 10 µg As/L did not differ, which is the result of the
24 leaky *arsR* promoter and read-through (Fig. 5B). Basal level of *arsR* expression is required
25 for the natural resistance system to function because of the nature of the *arsR* transcriptional
26 organization. Since ArsR is a transcriptional repressor which binds within its own promoter
27 DNA, no *arsR* transcription would take place without leakiness.

29 Discussion

30 The aim of this study was to simplify the application of bioreporter bacteria by developing an
31 internal calibration method. The idea was to infer analyte concentrations from combinations
32 of qualitative (yes/no) signals provided by a series of cell lines with different sensitivities for
33 the analyte. The quantitative response of a single cell line used so far in bioreporter assays is
34 replaced by a series of complementary qualitative responses. The advantage of such biosensor

arrays is their relative independence from incubation times and bioreporter activities. We used two different ways to produce such variable reporter cell lines, i) by influencing the rate of reporter enzyme synthesis (LacZ) or ii) by influencing the reporter enzyme specific activity (for CCP). Various other ways of obtaining similar effects can be imagined, for instance, by using different stabilities of reporter proteins (Andersen et al. 1998). We focused on an arsenite detecting bioreporter, for which a simple qualitative assay could be useful in field campaigns notably in Southeast Asia (Smith et al. 2000; British Geological Survey 2001).

LacZ-Bioreporters. Arsenite measurements have been performed before with LacZ bioreporters under control of the *ArsR* promoter using classical spectrophotometric, chemiluminescence and electrochemical detection (Scott et al. 1997; Ramanathan et al. 1998), or by chromogenic visualization using X-gal (Stocker et al. 2003). We previously showed that leaky expression from the *arsR* promoter must be reduced to obtain gradually intensifying blue colour formation from X-gal as a function of arsenite concentration. This was accomplished by placing a second *ArsR* DNA binding site downstream of *arsR* (Stocker et al. 2003). Upon long incubation times, however, cells with this bioreporter construct (i.e., strain 1595) will intensify their blue colour at all arsenite concentrations, reducing the visibility of a good colour scale. We therefore produced one variant of strain 1595 in which the background is further reduced (strain 2066) by altering the ribosome binding site in front of *lacZ* (Fig. S1). Indeed, production of LacZ activity in strain 2066 is very low compared to the others, which was useful for the concept of the multi-strain assay, because the 2066 bioreporter only produces visible blue colour from X-gal at arsenite concentrations above 50 µg/L. At the engineered unique restriction sites in front of *lacZ* we then replaced fragments of various length originating from the wild-type *lacZ* promoter in *E. coli*, one of which (construct in strain 2245, Fig. S1) appeared to be suitable for the arsenite concentration range below 5 µg/L. Interestingly, strain 2245 reacted with visibly different blue colour formation from X-gal already at 0.2 µg/L arsenite, compared to the negative control. Thus, the responses of strains 2245 and 1595 complement each other very well in the low (0.2-5 µg/L) and medium (5-50 µg/L) concentration ranges. Although strain 1580 is not directly useful in showing colour differences at varying arsenite concentrations, it can be used as positive control for the quality of the test.

By probing *lacZ* mRNA levels in the four strains, we could provide evidence that the different LacZ activities in the reporter strains as a function of arsenite is due to both differential transcription efficiency or mRNA stability and translational effects. Two transcriptional effects were clearly seen: First, the inclusion of the secondary *ArsR* binding site in all

constructs except 1580, which reduced mRNA formation in the absence of arsenite and secondly, the lower *lacZ* mRNA abundance from the constructs based on plasmid pPROBE (i.e., 2066 and 2245), which might be due to a different plasmid copy number. However, the strong LacZ activity difference between 2066 and 2245 was not apparent from mRNA levels (Fig. 5B), but the result from the absence (2066) and presence (2245) of a good ribosome binding site. One can conclude that our intention to control the bioreporter sensitivity on the level of reporter enzyme synthesis was successful in that it led to a range of bioreporter strains of quite complementary sensitivities including a nearly constitutive control strain.

CCP-Bioreporters. The use of cytochrome c peroxidase as reporter gene resulted in a useful colouration as well, although the assay was a bit less robust than that for β -galactosidase. The main advantage of *ccp* was the availability of a well characterized set of mutants with variable activity against the classical peroxidase substrate guaiacol (Iffland et al. 2000; Iffland et al. 2001). In this case mRNA probing showed that all mutant *ccp* genes were transcribed with similar efficiencies, and as a function of arsenite concentration (except one, Fig. 5A). However, the colouration resulted in very different intensities at the same arsenite concentration for the various reporters. Those strains containing CCP with improved specific activity (1971, 1981, 1982) compared to the wild type (2031) gave high signals at very low arsenite concentrations, but with a relatively high background in the absence of arsenite. Their increased activity confirms previous findings of Wilming et al., who observed turnover numbers of 102 and 26 s⁻¹ of strain 1971 and 1982, respectively, compared with 0.35 s⁻¹ for the wild type enzyme (Wilming et al. 2002). The *ccp* gene in strain 1981 contains multiple mutations (Y39H-H60R-N184D-D217G-D224Y) and although the substrate turnover was reported to be only slightly higher [0.8 s⁻¹(Iffland et al. 2001)] than that of the wild type, a colouration similar to that of 1971 and 1982 for arsenite concentrations up to 100 μ g/L was observed. The CCP of the bioreporter strains with the lowest observed activity and sensitivity in our tests (2332, 2340) had higher reported turnover numbers (13 s⁻¹ and 5 s⁻¹, respectively) than the wild type enzyme (Wilming et al. 2002). The reasons for these discrepancies between reported enzyme activities and signals obtained in our experiments with whole cells are unknown. Measurements of enzyme activities in cell extracts were in accordance with the reported substrate turnover (supplementary Figure S1), which might be an indication for different enzyme activity in extracts and whole cells, or differences in substrate accessibility or transport in whole cells. Our results, however, show that it is possible to receive a set of bioreporter bacteria with varying sensitivity by modifying the catalytic activity of the reporter protein and to use combination of them for internal calibration.

Conclusions. We demonstrated that bioreporter bacteria of various sensitivities can be tailored by modifying different steps of the detection-signalling chain, i.e. the rate of reporter enzyme synthesis and the catalytic activity of the reporter protein. It appears thus possible to construct sets of bioreporter bacteria that, when combined, cover ranges of target concentrations that could not be covered by a single bioreporter organism due to restrictions of e.g. the induction factor, limitations of spectroscopic signal detection or the insensitivity of visual interpretation. Furthermore our results demonstrate the potential of reporter protein engineering as opposed to the former focus on analyte sensing and gene regulation for bioreporter improvement (van der Meer et al. 2004).

The accomplished modifications altogether resulted in two sets of bioreporter bacteria that can be used to infer analyte concentration ranges without external calibration. This quantitative information originates from combinations of robust qualitative signals of the individual bioreporter strains. The hypothesized working principle was thus shown to function. A further important result is that permissive concentrations of arsenic could be identified with both bioreporter sets: (i) below the WHO limit only two of the four LacZ bioreporter bacteria produced a visible signal, above 10 µg/L three or at concentrations above local arsenic standards (50 µg/L) all four strains were responding with clear signals; (ii) the CCP bioreporter bacteria indicate a concentration below the WHO limit when four out of six strains show signals. This set could also be reduced to four strains, since three strains gave redundant information.

Our approach promises to simplify bioreporter application by layman since it eliminates time consuming and technically demanding calibration. Especially for simple field detection devices such as paper strips (Stocker et al. 2003) our method would be of great value. Besides its higher robustness it has the advantage of the much easier interpretation of sets of qualitative sub-signals than of gradual colour changes.

Experimental Procedures

Construction of ArsR plasmids.

The following reporter plasmids were constructed (Fig. 6) in which ArsR controls expression of either the *lacZ* gene or the *ccp* gene [for cytochrome *c* peroxidase from *Saccharomyces cerevisiae* (Iffland et al. 2000)]. In plasmid pMV-arsR [strain number 1580, (Stocker et al. 2003)] the *arsR* gene is placed directly upstream of *lacZ* and in the same direction of transcription, so that expression of *arsR* and *lacZ* are under control of the ArsR-regulated

promoter in front of *arsR* itself (P_{ars} , Fig. 6). To reduce background *lacZ* expression from the leaky P_{ars} promoter, a second ArsR DNA binding site (ABS) was introduced downstream of *arsR* in plasmid pMV-arsR-ABS [strain number 1595, Fig. 6, (Stocker et al. 2003)]. To change the sequence and ribosome binding site upstream of *lacZ*, we amplified the entire *lacZ* gene from the *E. coli* K12 chromosome while introducing HindIII and NheI restriction sites at the gene extremities. This *lacZ* DNA was then used to replace the *gfp* gene in the broad host range vector pPROBE' (Miller et al. 2000). The *arsR* gene and the downstream secondary ArsR binding site were recovered on a BamHI-SpeI fragment and inserted into pPROBE'-*lacZ* in front of *lacZ* to make pPR-ArsR-ABS-LacZ (strain 2066, Fig. 6). The ribosome binding site (RBS) of the original *lacZ* gene of *E. coli* was amplified together with the entire *lacZ* gene by PCR by using primer LacZ Rev NheI (5' gctagctattttgacaccagaccaactgg 3') and primer LacZ RBS For (5' gcttgtgagcggataacaatttcacacagg 3') and introduced in pPR-ArsR-ABS-LacZ digested by HindIII and NheI (plasmid pPR-arsR-ABS-RBS-LacZ, strain 2245, Fig. 6). Sequences of the *arsR* – *lacZ* intergenic regions are presented in supplementary figure S2.

The *ccp* reporter plasmids were all constructed by inserting a BamHI-SpeI fragment containing *arsR* and the second ArsR binding site from pPR-arsR-ABS (Stocker et al. 2003) in a pET15-based plasmid containing the *ccp* gene or its variants (Iffland et al. 2000; Iffland et al. 2001), pre-digested with BglII and XbaI (during which the T7 promoter is removed). The *arsR* gene is thus expressed from P_{ars} and on its turn regulates *ccp* expression (Fig. 6). In this manner we produced *E. coli* strains with plasmids pArsR-CCP-WT (strain number 2031, wild-type *ccp*), pArsR-CCP-R48I (strain 2332, R48I mutation in CCP), pArsR-CCP-R48T (strain 2340), pArsR-CCP-R48Q (strain 1971), pArsR-CCP-K2.4 (strain 1981, Y39H-H60R-N184D-D217G-D224Y mutations) and pArsR-CCP-PT1 (strain 1982, R48H mutation).

Arsenite bioreporters were constructed in *E. coli* Dh5 α .

Cell preculturing. Arsenite in aqueous solution was measured with cell suspensions of *E. coli* strains (Tab. 2). Bioreporter strains were plated from -80°C stock cultures on fresh Luria-Broth (LB) plates with appropriate antibiotic selection (ampicillin 100 μ g/mL or kanamycin 50 μ g/mL) and grown overnight at 37°C. Five mL liquid LB medium plus antibiotic was inoculated with a single colony and incubated for 16 h at 37°C. Cultures were diluted in fresh preheated LB medium without antibiotics at a 1:50 ratio and incubated until an optical density at 600 nm of 0.5 was reached. With this culture triplicate bioassays were performed as described below.

Arsenite assays with *E. coli* β -galactosidase (LacZ) bioreporters. Assay mixtures containing 100 μ L cell suspension (OD_{600} of 0.5, 1:5 diluted in water), 90 μ L arsenite stock solution and 10 μ L X-gal substrate solution (5-bromo-4-chloro-3-indolyl- β -D-galactosidase, Serva, 0.2 mg/mL in dimethylformamid) were prepared directly in 96-well plates. Cells were exposed to different final arsenite concentrations (0, 0.05, 0.2, 0.5, 2, 4, 6, 10, 20, 25, 30, 40, 50, 60, 70, 80 and 100 μ g/L). Arsenite solutions were prepared by dilution from a 50 mM (6.5 g/L) sodium arsenite solution commercial stock (Merck) in tap water. The 96-well plates with the assay mixtures were covered with a lid and incubated at 30°C in a rotary shaker (700 rpm) for 22 hours. In order to detect the blue colour formed from X-gal conversion, the optical density was measured in a microplate reader (Spectramax 250, Molecular Devices) at 595 nm after 0, 1, 2, 3, 4, 5, 6, 7, 8 and 22 h. The turbidity resulting from the cell suspension itself was subtracted from the measured signal by measuring triplicate assay mixtures without X-gal.

Arsenite measurements with *E. coli* cytochrome c peroxidase (CCP) bioreporters. The CCP bioreporters were cultured as above for the LacZ reporters in small precultures for 16 h at 37°C after which cells were diluted fiftyfold and again grown until a culture turbidity of \approx 0.5 was reached. Cells were then concentrated by centrifugation to achieve a turbidity at 600 nm of \approx 6. Ten μ L of these cell suspensions were then spotted on agar plates containing different concentrations of arsenite (0, 1, 4, 10, 20, 30, 50, 100, 200, 1000 and 5000 μ g/L) and briefly dried. After an incubation of four hours at 37°C the cell spots were transferred to paper by gently pressing a paper sheet (3M chromatography paper, Whatman) of the right diameter directly on the agar surface. Peroxidase activity of the cells was detected by incubating the paper sheets on filter paper which was wetted with 50 mM potassium phosphate buffer (pH 6.0) containing freshly added guaiacol (final concentration 105 mM, Sigma) and hydrogen peroxide (final concentration 170 μ M, Merck) (Iffland et al. 2000). Colour development was allowed to proceed until clearly visible, after which the paper sheets were digitally photographed.

Determination of mRNA contents. Reporter strain cultures grown similarly were exposed to 0, 10 or 100 μ g/L arsenite for 4 h at 37°C, after which total RNA was isolated from 5 ml culture using a Macherey Nagel Nucleo Spin Kit. Twenty μ L of 120 μ L total RNA extract from each sample was blotted on a positively charged nylon membrane (Roche) using a Milliblot system (Millipore). The total RNA on the membrane was fixed with UV (1 min at 120 mJ/cm²) and stored at room temperature until hybridization. DIG labelled DNA probes were synthesized using random primed labelling (DIG DNA labelling kit, Roche) from PCR

1 products generated with specific primers (Tab. 3) for *lacZ* and *ccp*, respectively.
2 Hybridization was performed for 16 h at 57°C for the *ccp*- and 53°C for the *lacZ*-probe using
3 DIG Easy Hyb solution (Roche) and 100 ng/μL probe. Subsequent washing was performed
4 according to the manufacturer's protocol (Roche). DIG-DNA-RNA hybrids were detected
5 with the NBT-CSIB (Roche) approach as described by the manufacturer. Dot blots were
6 photographed and quantitatively analyzed using Image J, in which the signal intensities (i.e.
7 the colour intensity of the dots) were expressed as average grey values.

8
9 **Acknowledgements.** The authors thank Kai Johnsson for the generous gift of the *ccp*
10 variants. Alexandra Baumeyer is thanked for her help in *arsR-ccp* cloning and preliminary
11 tests with cytochrome c peroxidase.

Table 1: Arsenite concentration ranges distinguished with combinations of LacZ-strains (A) and CCP-strains (B). Strains that give a signal in the respective concentration range are marked with x.

A	Concentration range	1580	1595	2245	2066		
	< 6 µg/L	x		x			
	10 – 50 µg/L	x	x	x			
	> 50 µg/L	x	x	x	x		
B	Concentration range	1971	1981	1982	2031	2332	2340
	0 µg/L	x	x	x			
	1 – 10 µg/L	x	x	x	x		
	> 20 µg/L	x	x	x	x	x	x

1 Table 2: Identification and characteristics of *E. coli* bioreporter constructs (ABS: ArsR
2 binding site, RBS: ribosome binding site).

3	Strain (LacZ)	Plasmid
4	1580	pMV-arsR
5	1595	pMV-arsR-ABS
6	2245	pPR-arsR-ABS-RBS-lacZ
7	2066	pPR-arsR-ABS-lacZ
8	Strain (CCP)	
9	1971	pArsR-ABS-CCP-R48Q
10	1981	pArsR-ABS-CCP-K2.4
11	1982	pArsR-ABS-CCP-PT1
12	2031	pArsR-ABS-CCP-WT
13	2332	pArsR-ABS-CCP-R48I
14	2340	pArsR-ABS-CCP-R48T

15

1 Table 3: Primers for the synthesis of probes for dot blot hybridization.

2 Primer name Target Sequence

3 sfi_for *ccp* 5'-GCC ATG GCC AGC ACG GCC ACA CC-3'

4 R48Trev *ccp* 5'-GTG CCA AGC AAG AGT GAC TAA TAC GGG-3'

5 LacZfor *lacZ* 5'-TCG GTT ACG GCC AGG ACA GT-3'

6 LacZrev *lacZ* 5'-CAT CAT TAA AGC GAG TGG CAA CAT-3'

7

Figure Captions

Figure 1: Principle of the internal calibration approach. A – D represent bioreporter cell lines differing in their sensitivity to arsenite.

Figure 2: Colour development after 480 min by four bioreporter strains expressing LacZ measured as absorbance at 595 nm. Data points represent the average of three replicates.

Figure 3: Colour development from X-gal by four LacZ-based bioreporter strains as response to different arsenite concentrations. A) Wide concentration range overview of assay carried out in 96-well plates. The digital image was taken after 8 hours, whereas colour development proceeded for 22 hours. B) Colour detail in assays performed in 24-well plates. Image taken after 4 hours incubation time at 30°C.

Figure 4: Colour development in the six bioreporter strains expressing CCP as a function of arsenite concentration in the assay. Colonies were spotted on paper after 4 hour incubation with arsenite on LB-agar plates at 37°C. Digital image was taken 10 min after adding guaiacol and hydrogen peroxide to develop colour.

Figure 5: Comparison of signal intensities after dot blot hybridization of mRNA in the six bioreporter strains expressing CCP (A) and in four bioreporter strains expressing LacZ (B) induced with three different arsenite concentrations for four hours.

Fig. 6: Schematic picture of the plasmids developed in this study. Genes are represented as arrows, drawn to proportion. Relevant restriction sites for cloning are shown in their usual abbreviations. The black vertical bars symbolize the ArsR binding sites (ABS).

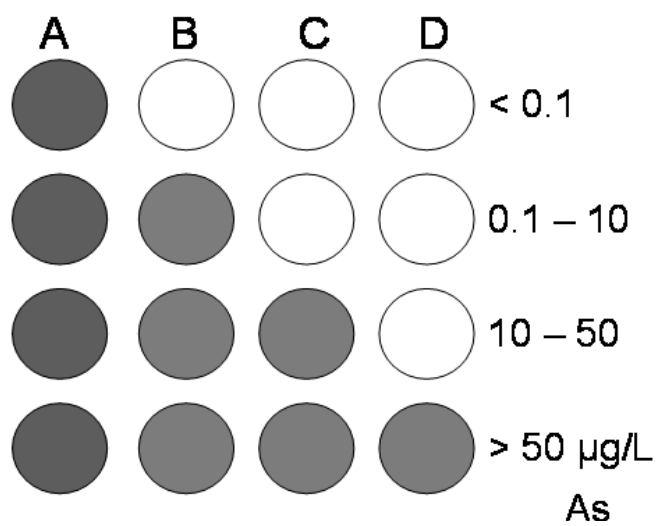
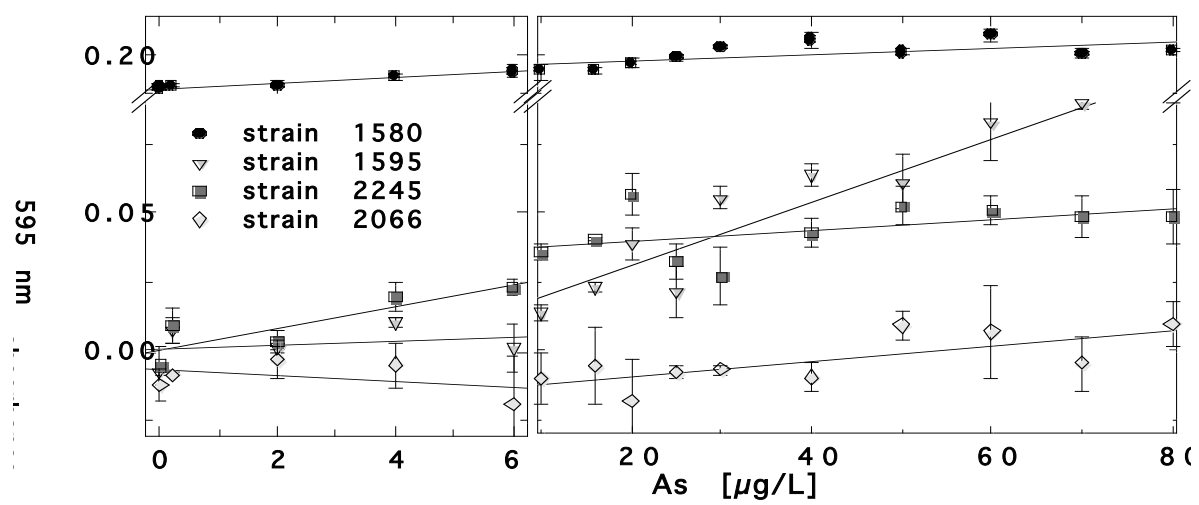


Fig. 1

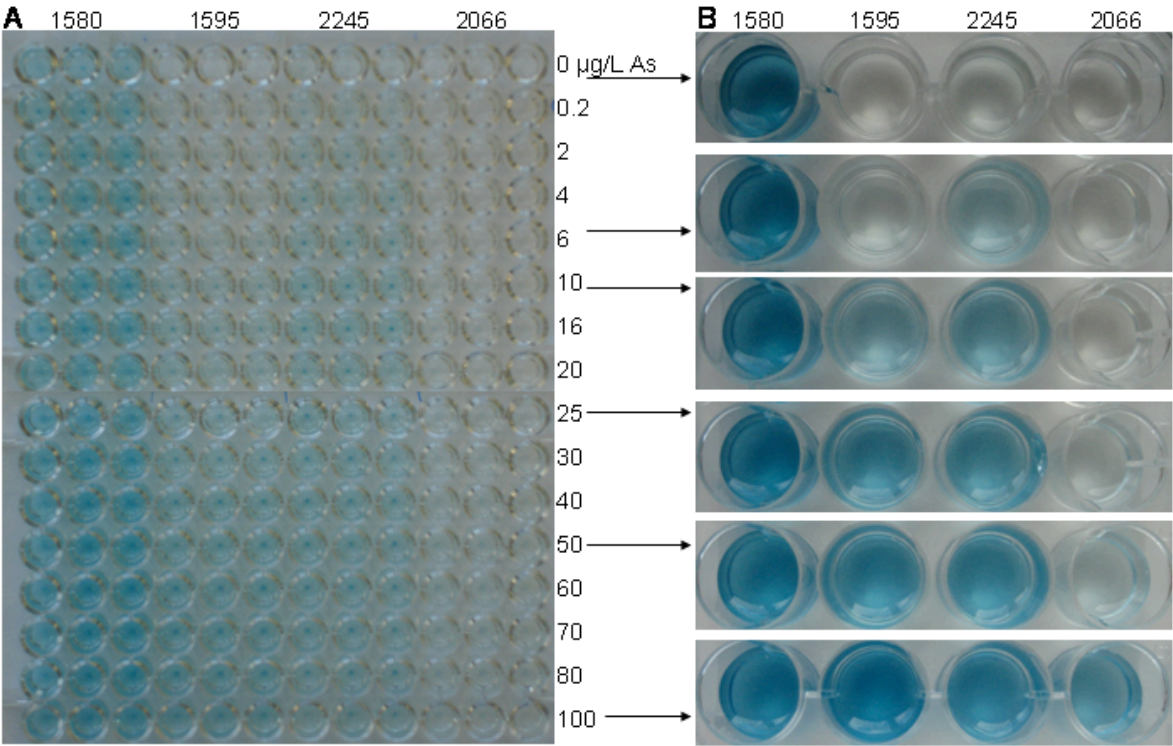
1 Figure 2



2

3

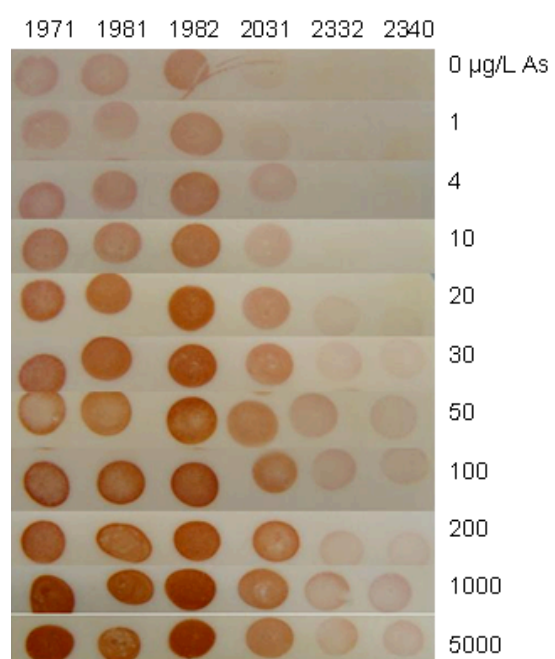
1 Figure 3



2

3

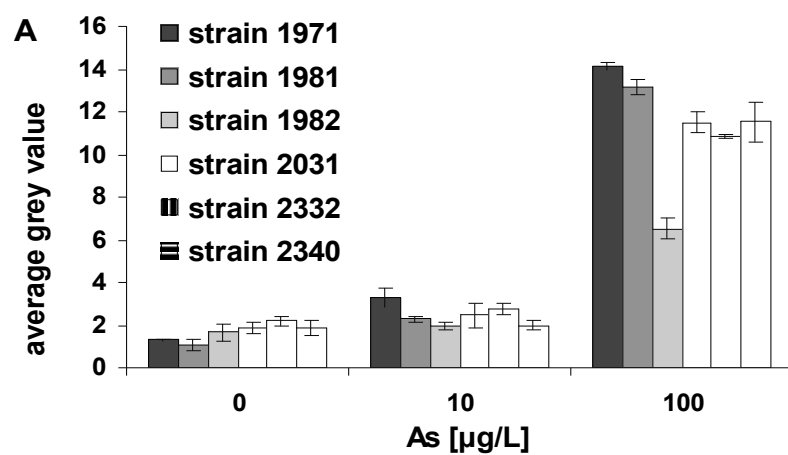
1 Figure 4



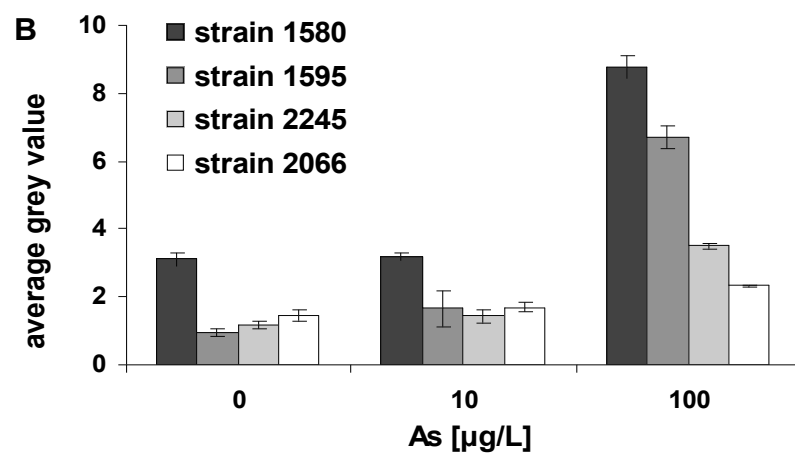
2

3

1 Figure 5



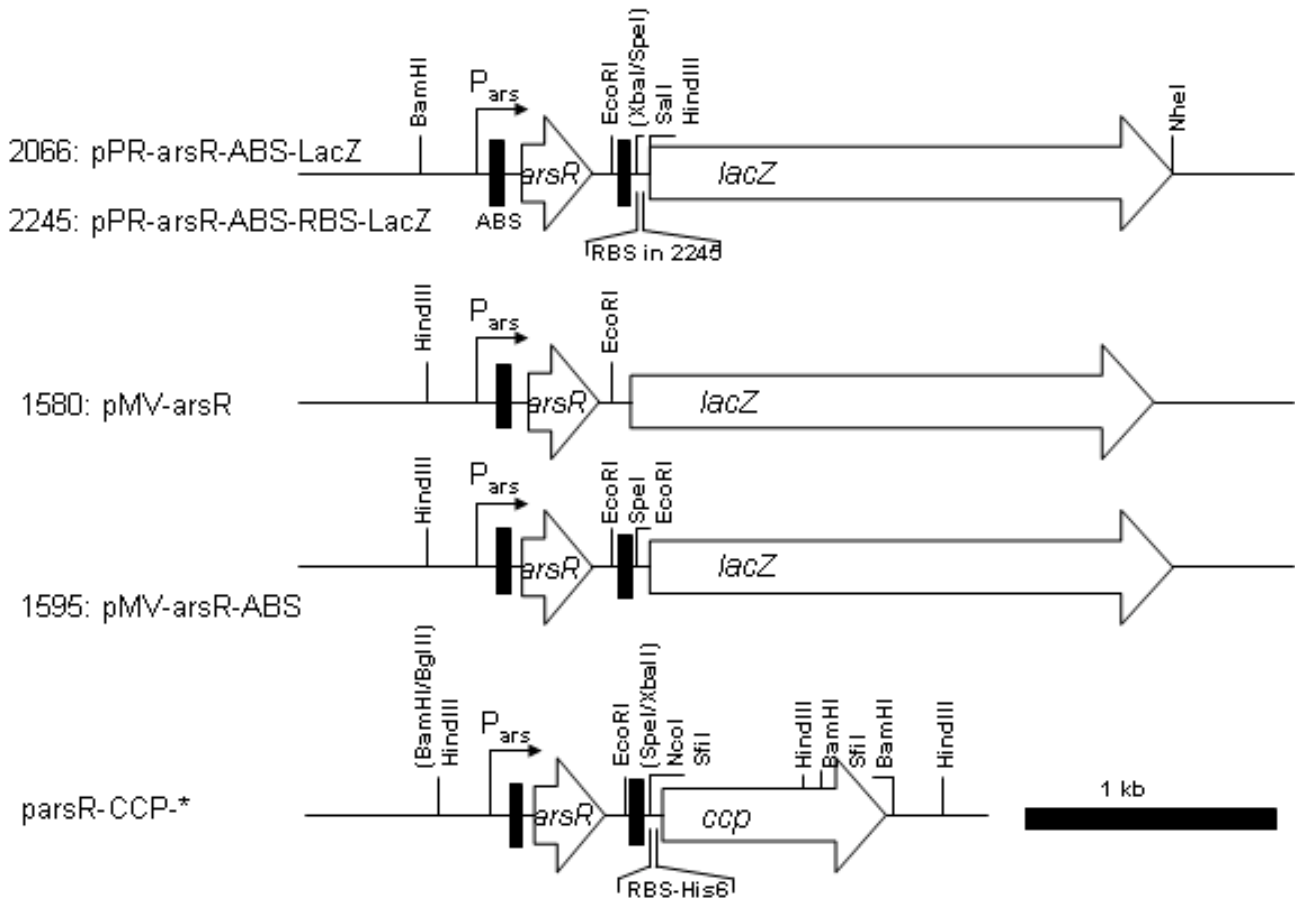
2



3

4

1 Figure 6



2

3

References

- Andersen, J. B., C. Sternberg, L. K. Poulsen, S. P. Bjorn, M. Givskov and S. Molin (1998). "New Unstable Variants of Green Fluorescent Protein for Studies of Transient Gene Expression in Bacteria." *Appl Environ Microbiol* **64**: 2240-2246.
- Belkin, S. (2003). "Microbial whole-cell sensing systems of environmental pollutants." *Curr Opin Microbiol* **6**: 206-212.
- Arsenic contamination of groundwater in Bangladesh. BGS Technical Report WC/00/19; British Geological Survey: London, 2001
- Daunert, S., G. Barrett, J. S. Feliciano, R. S. Shetty, S. Shrestha and W. Smith-Spencer (2000). "Genetically engineered whole-cell sensing systems: Coupling biological recognition with reporter genes." *Chemical Rev* **100**: 2705-2738.
- DeAngelis, K. M., P. S. Ji, M. K. Firestone and S. E. Lindow (2005). "Two novel bacterial biosensors for detection of nitrate availability in the rhizosphere." *Appl Environ Microbiol* **71**: 8537-8547.
- Hakkila, K., T. Green, P. Leskinen, A. Ivask, R. Marks and M. Virta (2004). "Detection of bioavailable heavy metals in EILATox-Oregon samples using whole-cell luminescent bacterial sensors in suspension or immobilized onto fibre-optic tips." *J Appl Toxicol* **24**: 333-342.
- Harms, H., J. Rime, O. Leupin, S. J. Hug and J. R. van der Meer (2005). "Effect of groundwater composition on arsenic detection by bacterial biosensors." *Microchim Acta* **151**: 217-222.
- Harms, H., M. C. Wells and J. R. van der Meer (2006). "Whole-cell living biosensors - are they ready for environmental application?" *Appl Environ Biotechnol* **70**: 273-280.
- Iffland, A., S. Gendreizig, P. Tafelmeyer and K. Johnsson (2001). "Changing the substrate specificity of cytochrome c peroxidase using directed evolution." *Biochem Biophys Res Commun* **286**: 126.
- Iffland, A., P. Tafelmeyer, C. Saudan and K. Johnsson (2000). "Directed molecular evolution of cytochrome c peroxidase." *Biochemistry* **39**: 10790-10798.
- Ivask, A., M. Virta and A. Kahru (2002). "Construction and use of specific luminescent recombinant bacterial sensors for the assessment of bioavailable fraction of cadmium, zinc, mercury and chromium in the soil." *Soil Biol Biochem* **34**: 1439.
- Jansson, J. K. (2003). "Marker and reporter genes: illuminating tools for environmental microbiologists." *Curr Opin Microbiol* **6**: 310.
- Lei, Y., W. Chen and A. Mulchandani (2006). "Microbial biosensors." *Anal Chim Acta* **568**: 200.
- Leveau, J. H. J. and S. E. Lindow (2002). "Bioreporters in microbial ecology." *Curr Opin Microbiol* **5**: 259-265.
- Miller, W. G., J. H. J. Leveau and S. E. Lindow (2000). "Improved *gfp* and *inaZ* broad-host-range promoter-probe vectors." *Mol Plant Microbe Interact* **13**: 1243-1250.
- Paitan, Y., I. Biran, N. Shechter, D. Biran, J. Rishpon and E. Z. Ron (2004). "Monitoring aromatic hydrocarbons by whole cell electrochemical biosensors." *Anal Biochem* **335**: 175-183.
- Ramanathan, S., W. P. Shi, B. P. Rosen and S. Daunert (1998). "Bacteria-based chemiluminescence sensing system using beta-galactosidase under the control of the ArsR regulatory protein of the ars operon." *Anal Chim Acta* **369**: 189-195.
- Scott, D. L., S. Ramanathan, W. P. Shi, B. P. Rosen and S. Daunert (1997). "Genetically engineered bacteria: Electrochemical sensing systems for antimonite and arsenite." *Anal Chem* **69**: 16-20.

- 1 Smith, A. H., E. O. Lingas and M. Rahman (2000). "Contamination of drinking water by
2 arsenic in Bangladesh: a public health emergency." *Bull World Health Organ* **78**: 1093 -
3 1103.
- 4 Stocker, J., D. Balluch, M. Gsell, H. Harms, J. Feliciano, S. Daunert, et al. (2003).
5 "Development of a set of simple bacterial biosensors for quantitative and rapid
6 measurements of arsenite and arsenate in potable water." *Environ Sci Technol* **37**: 4743-
7 4750.
- 8 van der Meer, J. R., D. Tropel and M. Jaspers (2004). "Illuminating the detection chain of
9 bacterial bioreporters." *Environ Microbiol* **6**: 1005-1020.
- 10 Wells, M., M. Gosch, R. Rigler, H. Harms, T. Lasser and J. R. van der Meer (2005).
11 "Ultrasensitive reporter protein detection in genetically engineered bacteria." *Anal Chem*
12 **77**: 2683-2689.
- 13 Werlen, C., M. C. M. Jaspers and J. R. van der Meer (2004). "Measurement of biologically
14 available naphthalene in gas and aqueous phases by use of a *Pseudomonas putida*
15 biosensor." *Appl Environ Microbiol* **70**: 43-51.
- 16 Wilming, M., A. Iffland, P. Tafelmeyer, C. Arrivoli, C. Saudan and K. Johnsson (2002).
17 "Examining reactivity and specificity of cytochrome c peroxidase by using combinatorial
18 mutagenesis." *Chembiochem* **3**: 1097-1104.
- 19
20
21

Supplementary Material

Experimental Procedures

Measurement of cytochrome c peroxidase (CCP) activity in induced arsenite bioreporters. The CCP bioreporters were cultured in 2 mL LB medium plus antibiotic for 16 h at 37°C, diluted fifty fold and grown again until OD₆₀₀ of 0.5 was reached. The cultures were then incubated for 4 hours at 37°C with arsenite concentrations of 0, 10, 30, 50 and 80 µg/L. After centrifugation of 1.5 ml cell culture at 4500 rpm for 5 min, cell pellets were resuspended in 1 ml of potassium phosphate buffer (50 mM, pH 6.0) and stored on ice until cell lysis. Cell lysis using sonication was performed with 30 W at intervals of 0.5 sec for 4 min (Labsonic U, B. Braun Biotech International GmbH). 100 µl of this cell extract were mixed with 100 µl potassium phosphate buffer in a well of a 96-well plate. The substrate guaiacol (10 mM) and hydrogen peroxide (1.4 mM) were added and the enzyme kinetic was measured in a plate reader at 470 nm for 4 min. Measurements were conducted in triplicates. Protein content of the samples was established using the Bradford method.

1 **Figure Captions**

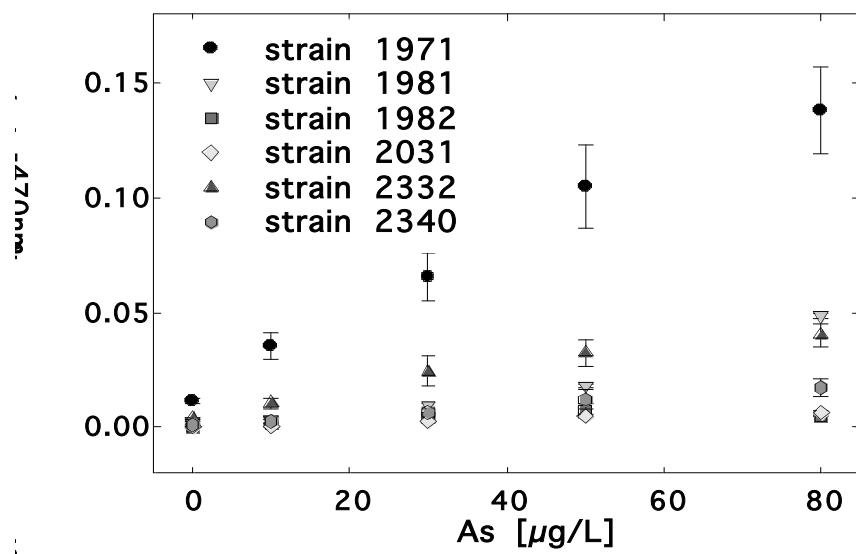
2

3 Figure S1: Specific activity of CCP in cell extracts from CCP bioreporter bacteria incubated
4 for 4 h with five different arsenite concentrations.

5

6 Figure S2: Intervening sequences in the *arsR-lacZ* constructions between the end of *arsR* (*taa*,
7 underlined) and the beginning of *lacZ* (*atg*, underlined). Relevant restriction sites used for
8 cloning are indicated. The *ArsR* binding site motif is shaded in grey background. RBS,
9 ribosome binding site. Plasmid pBGD23 is the original construct used by Scott et al, 1997.

1 Figure S1



2

1 Figure S2

2 pBGD23
3 taaaaaatttgcctgaacatatatgttttatcaaagcgagggtatttaagatgaaaacggttaatggatatttgacccggcgatgtgttgc
4 stop arsR rbs start arsD
5 agcaccggcggtctgcggtacagatgttgatcagg
6 fusion to 9th codon of lacZ BclI
7
8 pMV-ArsR basis pMV132 (1580)
9 taaaaaatttgcctgaattccgatccgacaaccgatgaaagcggcgacgcgcagttaatcccacagccgccagttccgctggcggcatt
10 stop arsR EcoRI
11 ttaactttctttatcacacaggaacagct.atg
12 rbs start lacZ
13
14 pMV-ArsR-ABS basis pMV132 (1595)
15 taaaaaatttgcctgaattccaagttatctcacctaccttaaggtaatagtgtgattcatcatatgcggttttggttatgtgaattaat
16 stop arsR EcoRI ArsR binding site motif
17 cactagtgaattccgatccgacaaccgatgaaagcggcgacgcgcagttaatcccacagccgccagttccgctggcggcattttaactt
18 SpeI EcoRI
19 tctttatcacacaggaacagct.atg
20 rbs start lacZ
21
22 pPR-ArsR-ABS-lacZ (2066)
23 taaaaaatttgcctgaattccaagttatctcacctaccttaaggtaatagtgtgattcatcatatgcggttttggttatgtgaattaat
24 stop arsR EcoRI ArsR binding site motif
25 ctctagtgtcgacggaagctt.atg.acc.atg
26 (SpeI/XbaI)SalI HindIII start lacZ
27
28 pPROBE' arsR-ABS-RBS-LacZ (2245)
29 taaaaaatttgcctgaattccaagttatctcacctaccttaaggtaatagtgtgattcatcatatgcggttttggttatgtgaattaat
30 stop arsR EcoRI ArsR binding site motif
31 cactagagtcgactgcagaagcttgtgagcggataacaatttcacacaggaacagct.atg.acc.atg
32 HindIII rbs lacZ start lacZ
33